Supplementary Information

Multivalent Sgc8c-aptamer decorated polymer scaffolds for leukemia targeting

Zhaobao Zhang[a], *Chunling Tang*[b], *Roel Hammink*[c,d], *Frank H. T. Nelissen*[a], *Hans A. Heus**[a], *Paul H. J. Kouwer**[a]

a Institute for Molecules and Materials, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands.

b Radiotherapy & OncoImmunology Laboratory, Department of Radiation Oncology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Centre, Geert Grooteplein 26, 6525 GA Nijmegen, The Netherlands.

c Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Geert Grooteplein 26, 6525 GA Nijmegen, The Netherlands.

d Division of Immunotherapy, Oncode Institute, Radboud University Medical Center, Nijmegen, The Netherlands.

Materials and methods

PIC polymer synthesis and characterization. The synthesis of sgc8c aptamer-functionalized PIC polymers (A-PICs) includes two steps. The first step is the synthesis of PIC polymer; the second is the bioconjugation of sgc8c aptamers to the PIC polymer.

For the PIC polymer synthesis, the non-functional methoxy (OMe) terminated and the functional azide-terminated (N3) isocyanide monomers were synthesized as described. Azide-functionalized PIC (PIC-N3) polymers were obtained by copolymerization of two monomers, one carrying a functional azide group (N_3), the other an inert tetra(ethylene glycol) tail (OMe), see Figure 1. The synthesis of both monomers was described earlier.[1] For the polymerization, the monomers (ratio OMe to N_3 of 30:1) were dissolved in toluene in the presence of a $Ni(ClO_4)_2 \cdot 6H_2O$ catalyst (0.1 mg/mL in freshly distilled toluene/absolute ethanol 9:1), which statistically yields a polymer with a functional azide group every 3.5 nm. The final momnomer concentration was adjusted to 50 mg/mL and the mixture was stirred overnight before it was precipitated three times in diisopropyl ether. After the final precipitation, the polymer was dried and kept at -20 °C until use.

Molecular weight determination of PIC polymers is complex. Over the past years of PIC research in our lab, we have been unable to obtain meaningful GPC traces even with help of experts. Moreover, even with good separation, calibration would be troublesome. The highmolecular weigth prevent NMR-based end group analysis and MALDI-ToF measurements. Routinely, we use viscometry and AFM analysis to determine molecular weights. Typically, we find that the molecular weight obtained by viscometry levels off at low initiator concentrations (i.e. high molecular weight). In this regime AFM is a stronger technique. For this paper, the molecular weight was indeed determined from AFM experiments (Figure S1). Analysis gave a contour length L of the polymers was determind by atomic force microscopy[2] and gave L = 335 nm, corresponding to a molecular weight M_w = 1148 kDa.[3,4] In addition, we measured the intrinsic viscosity (Figure 2), which in our experience underestimates the molecular weight of long PICs. Here we find $[\eta]$= 12.39 mL/mg, which corresponds to M_v = 495 kDa, using the Mark-Houwink equation: $[\eta] = KM_v^a$ with Mark-Houwink parameters $K = 1.4 \cdot 10^{-9}$ and a = 1.75. These numbers have been determined for other polyisocyanides, but not for the class of PICs discussed in this manuscript (for which GPC data is lacking). Note that M_v strongly depends on the empirical parameter a, for which the persistence length of the polymer is important.

Polymer characterization (circular dichroism, UV-vis and FTIR, Figure S3A-C) are in line with earlier reports.[3,4] In addition, the ^{1}H NMR spectrum (PIC-N_3 in $CDCl_3$, Figure 3D) gives broad peaks only and cannot be used for meaningful analysis.

Functionalization of PIC polymer with sgc8c aptamer. To obtain A-PICs, the sgc8c DNA aptamer was conjugated to PEG4-DBCO, which can be linked to the PIC azides through the fast and efficient strain-promoted azide-alkyne cycloaddition (SPAAC) reaction. The sgc8c aptamer was purchased from Integrated DNA Technologies with a 5'NH_2 functional group (5'-/5 AmMC6/TTT TTT ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TTA GA -3'). In addition, a fluorescent dye-conjugated sgc8c DNA aptamer DNA with 3'6-FAM™ was also purchased for fluorescence microscopy imaging experiments (5'-/5 AmMC6/TTT TTT ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TTA GAT TTT TT/3 6-FAM -3'). The 5' amino modifier C6 (MC6) was used for conjugation with an activated NHS ester. Prior to use, aptamers were purified using 12% preparative denaturing polyacrylamide gel electrophoresis [2]. The DNA was electroeluted from the gel in an Elutrap device (Schleicher & Schuell)and desalted by ethanol precipitation. For the 6-FAM labeled aptamer, the purification procedure was carried out in the dark.

To equip the DNA with the strained alkyne azadibenzylcyclooctyne (DBCO), the sgc8c aptamer (500 μM) was reacted with DBCO-PEG4-NHS (1.25 mM, Jena Bioscience) in borate buffer (50 mM, pH 8.4) at 25 °C for 2 h. Subsequently, the product was purified using Illustra Microspin G-25 columns (GE Healthcare). The final yield of DNA was determined from the absorbance signal at 260 nm using a Nanodrop fibre-optic spectrophotometer (ND-1000, Thermo Scientific).

To a stock solution of PIC polymer (5 mg/mL, corresponding to 13.9 mM monomer concentration) containing 1/30 azide-functionalized monomer (0.46 mM N_3) in Milli-Q water was added DBCO-functionalized sgc8c aptamer (ultimate concentrations 125 μM DNA, 6.35 mM PIC monomer with 208 μM N_3) in phosphate buffered saline (PBS, pH 7.4) and the mixture was incubated at 25 °C for 12 h. The combination of a very high conversion found for the SPAAC reaction (commonly >90% yield) [2] and the access of azide groups in the reaction (ratio N_3 to DNA 1:0.6) ensures that virtually all DNA will be consumed and that the obtained A-PICs are ready to use without further purification.

A-PIC conjugation efficiency. The aptamer conjugation efficiency was quantified with both gel electrophoresis and a 3-azido-7-hydroxycoumarin dye assay.[2] For the PAGE gel electrophoresis, a serial dilution of sgc8c (100 ng, 200 ng, 300 ng, 400 ng) and A-PIC were loaded on the gel (Figure S4A). Gels (15% polyacrylamide) were ran for 1.5 h at 120 V, then stained with ethidium bromide (Sigma-Aldrich) for 10 min and scanned on Bio-Rad Gel Documentation System. The intensities were quantified by Image Lab software. Due to the large molecular weight of PIC, A-PIC remains stuck on top of the gel, however, unconjugated sgc8c runs similarly to unconjugated free sgc8c in the dilution series.

For the 3-azido-7-hydroxycoumarin dye assay, a calibration curve was first established to correlate the fluorescence intensity to the amount of clicked product; then the amount of unreacted DBCO, determined before and after incubation with the polymer (12 hours) were measured, which was used to calculate the reaction efficiency. Both analyses yield a degree of substitution of ~90 %.

Nuclease stability of A-PIC conjugate. Both free sgc8c and A-PIC were incubated in 1× PBS buffer supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich) at 37 °C.[5] After 12h and 24h incubation, samples were removed from the waterbath, mixed 1:1 with loading dye and run on a 15% polyacrylamide gel at 120 V for 1.5 h. Gels were then stained with ethidium bromide (Sigma-Aldrich) for 10 min and scanned on Bio-Rad Gel Documentation System. Band intensity was quantified with the Image Lab software.

CCRF-CEM leukemia cell culture. The CCRF-CEM leukemia cell line was purchased from ATCC® (CCL-119™). CCRF-CEM leukemia cells are human T lymphoblasts isolated from acute lymphoblastic leukemia patients. Upon arrival, the vial was thawed by gentle agitation in a 37 °C water bath, which took approximately 2 minutes. Then the cell suspension was centrifuged at 125g for 10 minutes and the cell platelets were resuspended in culture medium (RPMI 1640 medium, American Type Culture Collection, supplemented with 10% fetal bovine serum (Invitrogen) and 0.5 mg/mL penicillin-streptomycin). A T25 flask with resuspended cells was incubated horizontally at 37 °C in a 5% CO_2 incubator. The medium (50% volume) was refreshed every 3 days and a cell density of 500,000 viable cells/mL was kept in culture. Cells were washed before and after incubation with 1 x PBS buffer. Cell numbers were counted with TC20™ Automated Cell Counter (Bio-Rad) in 1:1 ratio with Trypan blue. Only viable cells were counted in all experiments unless otherwise stated. In a 6-well plate set-up, 125 mM A-PICs were added to each well (about 2 x 10^5 cells, Figure 3), fully-mixed, and incubated for 2 d before flow cytometry profiling. In terms of the control groups, 125 mM PICs (PICs group) or 2.5 mM sgc8c (DNA group) were added respectively.

Confocal microscopy. At day 2 after seeding, CCRF-CEM leukemia cells were sub-cultured and 6-FAM-A-PIC was added to each of the 6 wells on the plate. The cells were incubated for 2 h, 4 h, and 8 h under standard conditions and subsequently studied by confocal microscopy. All cellular images were collected on a Leica SP8 confocal microscope and analyzed with Fiji software.

Cell cycle analysis by flow cytometry. A cell cycle analysis of CCRF-CEM leukemia cells with/without A-PIC treatment was performed by using propidium iodide staining (ab14083, Abcam) following the manufacturer's instructions. Briefly, following the 2-day incubation of the CCRF-CEM leukemia cells with/without A-PICs, the cultures were washed with 1 x PBS buffer and fixed with 1 mL 70% alcohol and 30% PBS mixture at 4 °C for 1 hour. The cells

were then incubated in the dark with PBS, 20 µg/ml propidium iodide (ab14083, Abcam) and 1% RNaseA for 30 minutes at 37 °C. The cell samples were resuspended in 1 x PBS buffer and analyzed using FACS Calibur flow cytometry. Flow cytometry analysis was performed using a CyAn™ ADP Analyzer instrument (Beckman Coulter). The results were analyzed using Flow-Jo ver. 9.2 software (Tree Star Inc., USA).

Quantification and Statistical Analysis. Data are expressed as mean ± standard deviation unless specified otherwise. Dataset statistics (GraphPad Prism software): one-way ANOVA, followed by Tukey's multiple comparisons test (Figure 3, 4c). Differences of $p > 0.05$ were considered statistically non-significant, while differences of $p < 0.05$ were considered statistically significant: **, $p < 0.01$, ***, $p < 0.001$. Sample sizes are specified in the figure legends.

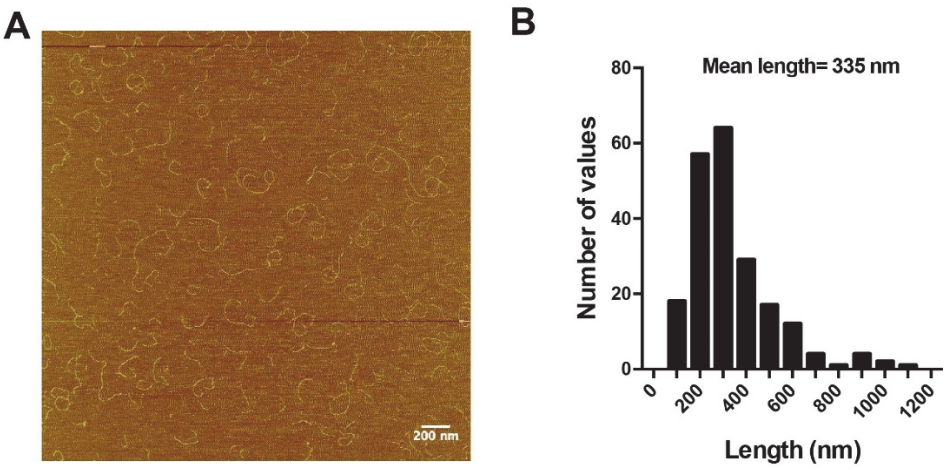

Figure S1. Atomic force microscopy (AFM) analysis to determine the PIC-N_3 polymer length.[2] (A) Representative AFM image of dilute aqueous PIC solution (1 µg/mL) as drop casted on freshly cleaved mica. Scale bar = 200 nm. (B) Histogram showing the polymer (contour) length and distribution from the AFM images. The data gives a mean polymer length of 335 nm, which corresponds to a molecular weight M_n = 1150 kDa.[3,4]

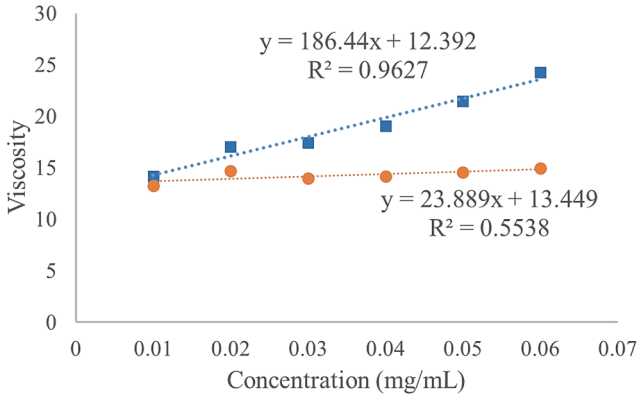

Figure S2. Ostwald viscosity measurement of PIC-N_3 in acetonitrile solution with reduced viscosity (blue squares) and intrinsic viscosity (orange dots). Extrapolation to zero concentration and averaging gives an intrinsic viscosity $[\eta]$ = 12.39 mL/mg, which corresponds to M_v = 495 kDa, using the Mark-Houwink equation: $[\eta] = KM_v^a$ with estimated Mark-Houwink parameters $K = 1.4 \cdot 10^{-9}$ and a = 1.75.

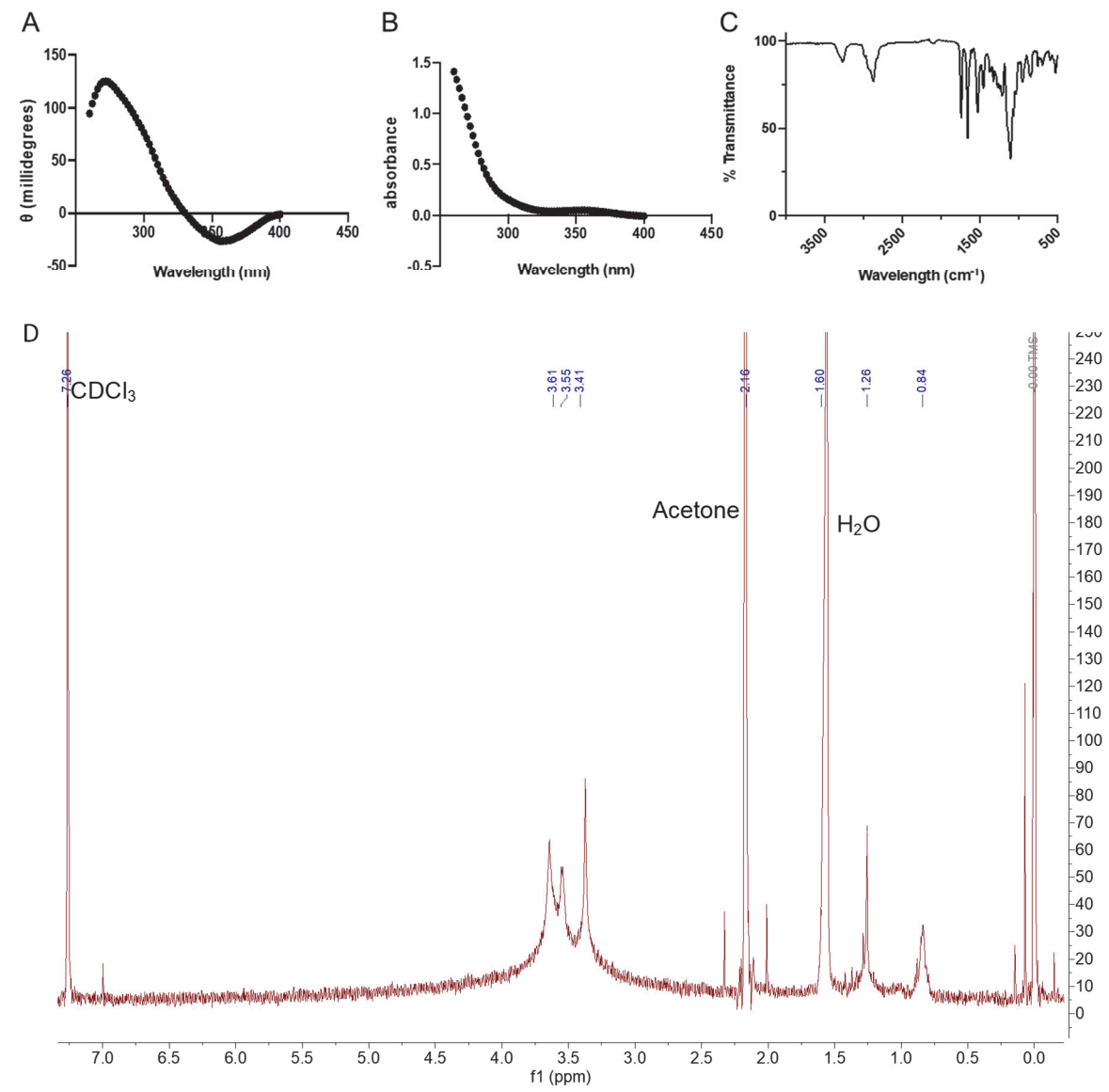

Figure S3. Additional PIC-N_3 characterization. (A) The circular dichroism spectra of the PIC-N_3 (0.226 mg/mL in water) shows a negative peak at λ = 360 nm and a larger postitive peak at λ = 270 nm, which are characteristic for the helical PIC main chain conformation.[3] (B) UV-vis spectrum (0.226 mg/mL in water) with a strong UV absorption of the main chain. (C) FTIR spectrum showing a characteristic (but small) azide peak at 2105 cm^{-1} is observed, confirming the presence of the functional handles. (D) The ^1H NMR spectrum of PIC-N_3 in $CDCl_3$ shows only broad signals of the ethylene glyocol tails and the alanine methyls.

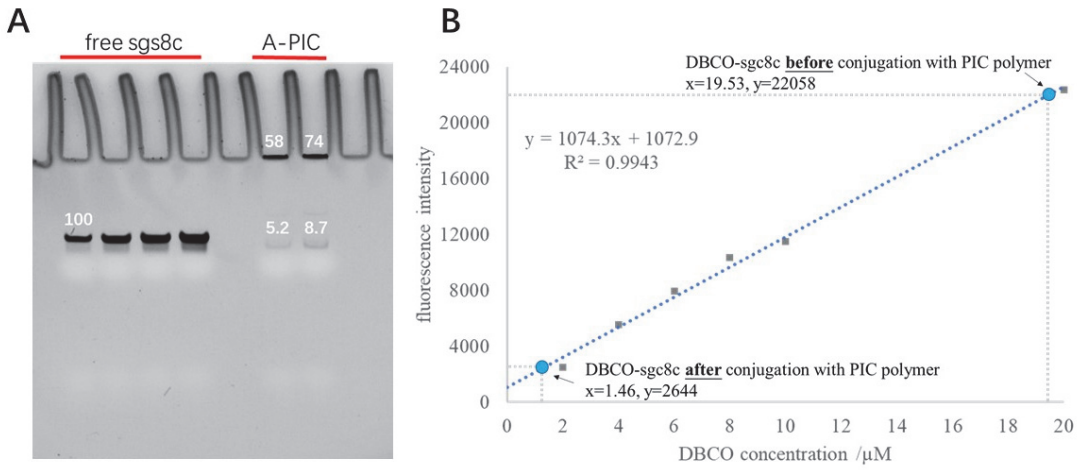

Figure S4. The characterization of A-PIC conjugate efficiency. A) Electrophoresis gel of free sgc8c and A-PIC conjugates (15% PAGE). A serial dilution of free sgc8c was loaded for the quantification of signal intensity, A-PICs were loaded in duplicates. The signal intensity was measured with Image Lab software and has been added next to the bands. A-PIC does not run on the gel as a result of high molecular weight. The coupling efficiency is ~90 %, calculated for both lanes: 58 / (5.2 + 58) * 100% = 91.7 % and 74 / (74 + 8.7) * 100% = 89.5 %. (B) Fluorescence intensity calibration curve of the SPAAC click-product of 3-azido-7-hydroxycoumarin with DBCO-functionalized sgc8c (grey data, dotted line is the linear fit) and experimental data points of before and after the reaction with the azide-functionalized PIC polymer after conjugation for 12 h (blue data). The reaction efficiency is calculated to be 92 % from (19.53 – 1.46)/19.53 *100% = 92 %.

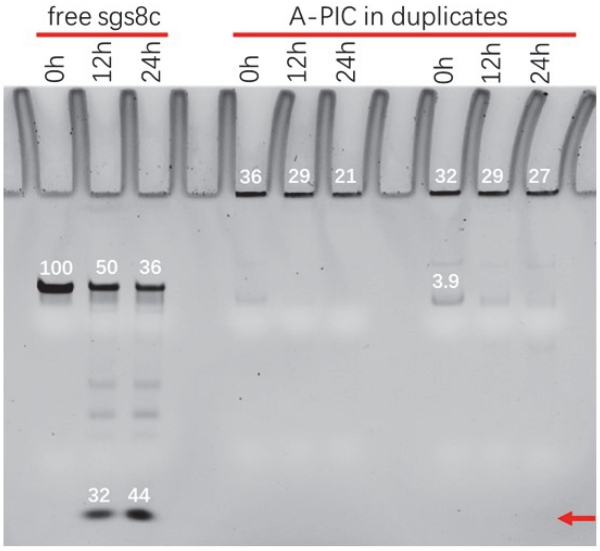

Figure S5. Degradation analysis of the free sgc8c aptamer and A-PIC after serum incubation (10 % FCS in PBS) by gel electrophoresis (15% PAGE). Signal intensities were measured with Image Lab software and are shown next to all the detectable bands.

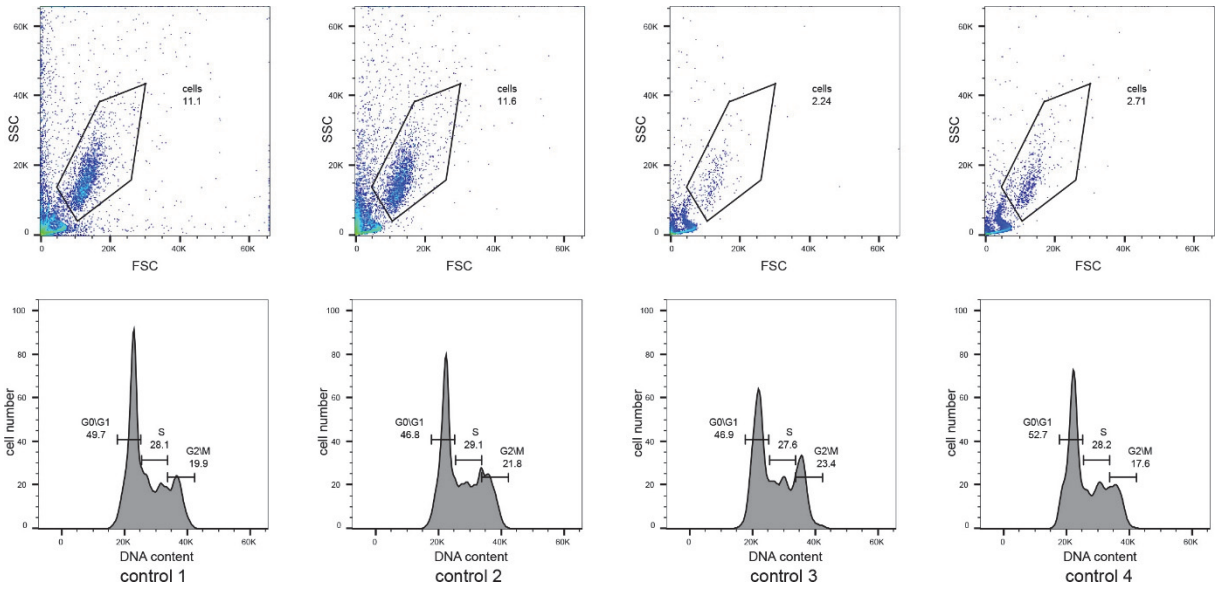

Figure S6. Flow cytometry histogram of cell cycle analysis of CCRF-CEM leukemia cell with 2-day PBS buffer incubation. The original results from four independent experiments are shown. The top bivariate histogram of forward scatter (FSC)/side scatter (SSC) analysis illustrated gates to eliminate debris and dead cells. In the bottom panel, X- and y- axes demonstrate DNA content and cell number, respectively. Each phase was calculated and the percentages of cells in G0/G1, S, and G2/M were also shown as indicated.

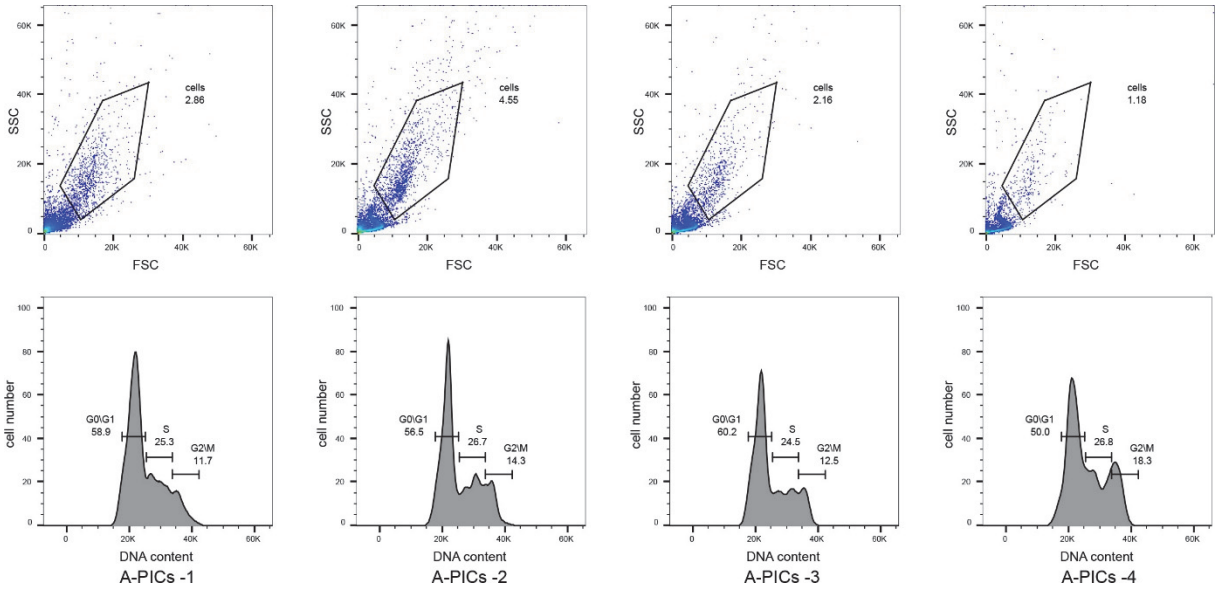

Figure S7. Flow cytometry histogram of cell cycle analysis of CCRF-CEM leukemia cell with 2-day A-PICs incubation. The original results from four independent experiments are shown. The top bivariate histogram of forward scatter (FSC)/side scatter (SSC) analysis illustrated gates to eliminate debris and dead cells. In the bottom panel, X- and y- axes demonstrate DNA content and cell number, respectively. Each phase was calculated and the percentages of cells in G0/G1, S, and G2/M were also shown as indicated.

References

1. S. Mandal, Z. H. Eksteen-Akeroyd, M. J. Jacobs, R. Hammink, M. Koepf, A. J. Lambeck, J. C. van Hest, C. J. Wilson, K. Blank and C. G. Figdor, *Chem. Sci.*, 2013, **4**, 4168-4174.
2. S. R. Deshpande, R. Hammink, R. K. Das, F. H. Nelissen, K. G. Blank, A. E. Rowan and H. A. Heus, *Adv. Funct. Mater.*, 2016, **26**, 9075-9082.
3. M. Koepf, H. J. Kitto, E. Schwartz, P. H. J. Kouwer, R. J. M. Nolte and A. E. Rowan, *Eur. Polym. J.*, 2013, **49**, 1510-1522.
4. P. H. J. Kouwer, M. Koepf, V. A. A. Le Sage, M. Jaspers, A. M. van Buul, Z. H. Eksteen-Akeroyd, T. Woltinge, E. Schwartz, H. J. Kitto, R. Hoogenboom, S. J. Picken, R. J. M. Nolte, E. Mendes and A. E. Rowan, *Nature*, 2013, **493**, 651-655.
5. L. Yang, M. Liang, C. Cui, X. Li, L. Li, X. Pan, H. Safari Yazd, M. Hong, J. Lu, Y. C. Cao and W. Tan, *ChemBioChem*, 2020, DOI: 10.1002/cbic.202000712, DOI: 10.1002/cbic.202000712.